USE OF BACTERIOCIN FOR THE AMELIORATION OF DIGESTIVE FUNCTIONALITY

DESCRIPTION

TECHNICAL FIELD

5   The subject of the present invention is the use of bacteriocins and/or their producer strains for the amelioration of digestive functionality and for the amelioration of gastrointestinal tract conditions in monogastric organism species.

10   ### STATE OF THE ART

Bacteria belonging to the genus *Pediococcus* are known. Bacteria belonging to the genus *Pediococcus* are homofermenting, facultative anaerobes non encapsulated. Bacteria belonging to the genus *Pediococcus* are 15   chemoorganotroph, therefore they have complex nutritive requirements, they don't have proteolytic activity and are saprophytae of many fresh and fermenting plants. Their economical importance in the transformation and conservation of plant products, meat and 20   their contribution in the development of cheese flavours make them a particularly attractive genus from the industrial point of view. Bacteria belonging to the genus *Pediococcus* have been isolated from bovine rumen and chick cecum. Eight species, depending on 25   their to sensitivity temperature, pH and NaCl may be identified.

P. cerevisiae species has been eliminated and previously thus indicated strains have met partly in P. damnosus species and partly in P. pentosaceus species. 30   Bacteria belonging to this latter class have their natural habitat in plants and exhibit development temperatures lower than 50°C (for FBB61 P. pentosaceus, the optimum is established at 35°C), in contrast to thermophile pediococci which grow well at 50°C, they

have smaller cell sizes (0,5-0,8 µm) and are grouped in P. acidilactici species.

It is known that one of the lactic bacteria features, which make them particularly interesting for a possible probiotic use, is their ability in competing with other bacteria for the same ecological niche, and to prevail. This ability is due to organic acids and metabolism by-products high concentrations production, assisted by substances with antimicrobial action proteic in nature, designated as "bacteriocins".

It is known that FBB61 Pediococcus pentosaceus microorganism is responsible for the production of a proteinaceous antimicrobial substance (bacteriocin) designated as pediocin A.

It is known that the lactic bacteria ability in competing with other bacteria for the same ecological niche and to prevail, besides of being due to organic acid production and low pH level tolerance, it is sometimes strengthened by the production of antimicrobial substances proteinaceous in nature. It is widely recognized that these proteins or peptides with antimicrobial action, designated with the term "bacteriocins" help in a decisive way to survival or dominance of certain strains in microbial ecosystems, such as foods or the digestive tract.

Exemples of bacteriocin productions exist in all known bacterial species and generally they only have an antagonistic activity against those species covering the same ecological niche or those philogenetically close to the producer microorganism. Lantionine-containing peptides such as nisin and subtilin constitute an exception to this rule, as they generally show antimicrobial activity against Gram +.

The very long and positive use of these bacteria

probably resides in the production of these bacterio-
cins till now studied for the purpose of extending
foods preservability and improving hygienic features
with reference to pathogenic microorganism develop-
5   ment.

Therefore, it is known that bacteriocins have been so
far studied, produced and purified for their possible
use like end products and/or raw materials preserva-
tives by virtue of their marked antimicrobial abili-
10   ties towards impairing and/or harmful microorganisms.
However, use of Bacteriocins as intestinal environment
modulators and sanitary conditions enhancers of the
intestine has not been investigated so far.

DESCRIPTION OF THE INVENTION

15   This invention relates to a new use of bacteriocins
and/or their producer strains as gastrointestinal
tract conditions enhancers of the monogastric organism
species here enclosed: humans, swines, rabbits, hor-
ses, poultry also wild, sheep, goats, felids, canids
20   and non functional ruminants. The addition of a
bacteriocin or its producer bacterium, a lactic bacte-
rium, in this case pediocin A and FBB61 *Pediococcus
pentosaceus*, FBB63 *Pediococcus pentosaceus* and its
bacteriocins, L 7230 *Pediococcus pentosaceus* and its
25   bacteriocins, to diet produces positive effects on the
organism thanks to a better general arrangement of the
gastrointestinal tract.

Lactic bacteria, including lactobacilli and bifid bac-
teria, regarded as GRAS (Generally Recognised as
30   Safe), which are introduced in the diet and/or admin-
istrated to organism lead to the production of lactic
acid inside the gastrointestinal tract, which deter-
mines an intestinal pH reduction. This low pH is in-
hospitable for several undesired microorganisms (in-

cluding human and animal phatogens) that cannot sur-
vive in so acidic conditions. Consequently, the inven-
tion developes a better microbic intestinal balance.
Colonization of these bacteria is promoted by the pro-
5.   duction of antimicrobial proteinaceous molecules,
called bacteriocins, which are able to act and there-
fore to be used in any way, alone as well, having a
self-activity, without the compulsory presence of pro-
ducer strain. A further advantage of the invention re-
10   sult from the reduction of oncogenic compounds due to
the proliferation of beneficial bacteria, either pro-
ducer strains of said bacteriocins or other beneficial
bacterial strains not affected by the antimicrobial
action of antimicrobial proteins. As it has been shown
15   by in vitro fermentation systems, such effects are
pointed out in the reduction of cresol production, a
tiroxina metabolite which is considered responsible
for the onset of skin and epatic tumors in mouse, as
well as a depressing factor of the animal growth, e.g.
20   in swines. Moreover, still into in vitro systems, the
presence of bacteriocins and/or their producer bacte-
rial strains leads to a considerable increase of poly-
amine productions of bacterial origin, especially pu-
trescine and spermidine, usually known as very impor-
25   tant energetic sources for gastroenteric cells. The
presence of high quantities of these molecules repre-
sents not only an important condition especially for
young animal, where the proliferation and renewal rate
of intestinal cells is higher than every other cell of
30   the organism. The above discussed remarks, pointed out
in vitro, show an actual modulation action of intesti-
nal metabolic processes by the bacterial component,
whose consequences find then expression in a higher
hygiene and health of gatroenteric tract, therefore a

better animal's state of health.

The introduction of bacteriocins and/or their producer strains into diet leads to a better morphofunctional arrangement of intestinal wall with an increase of
5   ephitelial surface deputed to the absorption of nutrients without affecting its integrity and consequently the functionality. These remarks together with a renewed and improved intestinal microbial balance and consequent metabolic modifications affect an optimal
10   organism state of health and a better efficiency of nutrients use. The efficiency of the invention also results from the interaction of cited bacteriocins with intestinal mucosa structures. More precisely, although not being related to any specific mechanism,
15   the descripted bacteriocins, when introduced with diet or produced in situ by respective producers bacterial strains play their action both in intestinal lumen and by being incorporate in mucous layer which cover the intestinal structures, and remaining active therein
20   they play a protection role against pathogenic bacteria attack, both human and animal, preventing the mucosa attack and the harmful action with consequent reduction of morbidity and onset of gastrointestinal disorders, detrimental to organism. Therefore, the in-
25   corporation in the intestinal mucus preserve the functional integrity and preserve the bacteriocins from digestive processes attacks. A surprising aspect of this invention is the gastroenteric environment modulation from all microbiologic, metabolic, hygienic,
30   sanitary and prophylactic aspects, with an amelioration of digestive tract health conditions. Therefore, the invention is particularly useful when bacteriocins and/or their producer strains are employed as additive, integrator, supplement into diet for all the

above mentioned species.

The use of invention further includes its employement in any extent in the prevention and prophylaxis of the pathologies by clostridia in any of the above men-
5 tioned species, as well as a support to any intervention before, during and/or after the onset of such pathologies.

In a preferred embodiment, this invention refers to a new use of bacteriocins and/or their producer strains
10 as enhancers of gastrointestinal tract conditions of monogastric organism species. Bacteriocins and/or more suitable bacterial strains are FBB61 *Pediococcus pentosaceus*, FBB63 *Pediococcus pentosaceus*, L 7230 *Pediococcus pentosaceus* (ATCC 43201) as well as all bacte-
15 riocins produced thereof and/or analogous molecules to pediocin A.

When ingested, microorganisms and/or their bacteriocins lead to an amelioration of food efficiency with a better use of nutrients and an improved microbiologi-
20 cal, metabolic, sanitary condition by way of: 1) lactic acid production with pH decrease and onset of adverse conditions for harmful and/or pathogenic bacteria, 2) gastroenteric tract colonization by beneficial bacteria at harmful microorganis' cost, 3)marked bac-
25 teriocins antimicrobial action against gram + bacteria, including Clostridia, adverse to organism, 4) metabolism modulation with reduction of the production of oncogenic products (cresol), 5) metabolism modulation with increase of the polyamines presence (putre-
30 scine and spermidine) into intestinal lumen, that function as important energetic sources for gastroenteric structures, 6) increase of intestinal absorptive surface and maintenance of its proper functionality, 7) adhesion of bacteriocins in the mucus covering gas-

troenteric mucosa with constitution of a protection
barrier against any harmful microorganisms action.
In a preferred embodiment, it is expected the intro-
duction of multiple bacteriocins producer bacteria

5   and/or the bacteriocins themselves in any useful com-
bination.
The addition and mixing of above mentioned components
may occur manually or by using any number and kind of
devices fitted for the manufacturing of small and

10   large quantities of liquid or solid feed. If the feed
is not particulated in nature, bacteria and bacterio-
cins may be added at the same time of granulation
and/or grinding and/or pulverization of the feed it-
self. Any method suitable for the feed preparation in

15   any form thereof may be used in order to associate and
make possible the introduction of bacteria and/or bac-
teriocins in the feed, also by using binding agent,
adsorbents, excipients and any other method and/or
substance suitable for the purpose.

20   Similarly, producer bacterial strains of bacteriocins
and/or their bacteriocins may be added to diet or to
single raw materials alone or in any combination, any
physical form both freeze-dried, liquid, dried, pel-
letized, chemical and free or protected form with any

25   proper method.
Conveniently, bacteriocins are present in the feed in
a quantity from 1 to 10,000 active units (AU) per ml
and/or g of feed. The invention further relates to the
use of bacteriocins producer strains administred in

30   any form and capable of producing such an amount of
bacteriocin that is included in the above mentioned
range.
The following examples are only to be intended as an
help for a better understanding of the invention. The

following examples do not limit in any way the dis-
cussed invention and/or the enclosed claims, neither
the applicability fields.

<u>EXAMPLES</u>

5   Bacterial strains and growth conditions
During the research they have been employed: FBB61
Pediococcus pentosaceus strain (ATCC 43200), as a
Pediocin A producer and FBB61-2 Pediococcus pento-
saceus strain, isogenetic mutant which does not pro-
10   duce bacteriocin and does not show immunity characters
(Imm-), (Daeschel, and Klaenhammer, 1985) as an indi-
cator strain. Those two strains were coltured over-
night, in M17 broth medium (Oxoid Ltd, Dasingstoke,
UK) + 1% (w/v) glucose at 39°C (Sambrook et al.,
15   1989). Agarized mediums and those used for overlay
tests were prepared with the addition of 1,5% (w/v)
agar to the liquid medium.

Bacteriocin production
The two FBB61 and FBB61-2 bacterial strains were incu-
20   bated in a M17 broth + 1% (w/v) glucose at 39°C for 24
hours. The coltures were then centrifugated at 10,000
rpm for 15 minutes and the supernatant collected and
filtered (Nalgene Filterware, Nalge Nunc Interna-
tional, NY, USA) through membranes with $\varnothing$ = 0,45 μm
25   pores. Next, the filtered supernatant was concentrated
through dialysis against polyethylene glycol of 20,000
Da molecular weight (PEG 20000, Sigma Chemical, St.
Louis, MO, USA). For this operation, dialysis tubes
with 12000/14000 MW cut-off (Spectra/Pro 4, Spectrum,
30   Houston, Texas, USA) were employed. The process was
carried out at a temperature of 4°C for a whole night.
The two solutions, in which the dialysis tube were
dipped, were constantly stirred by a stirrer, in order
to promote a better osmotic flow.

Pediocin A purification

The dialyzate of the two coltures FBB61 (Dia+ = dia-
lyzate of producer strain) and FBB61-2 (Dia- = dia-
lyzate of non producer and sensitive strain) was sub-
jected to further dialysis against a 150 mM NaCl, 20
mM Tris-HCl solution, still at a temperature of 4°C
overnight. The pH of the obtained solution was then
adjusted at 8,1 by addition of HCl 33%. The obtained
solution was further sterilized by way of filtration
through 0,45 μm porosity filters.

The pediocin A purification was carried out by low
pressure ion-exchange chromatography.

Five 5 ml each ion-exchange chromatographic columns
were mass-mounted and connected through silicone tubes
to a peristaltic pump (Minipuls 3, Gilson Italia Srl,
Milan, Italy). The thus obtained column was prepared
by first passing through it by a start-buffer (20 mM
Tris-HCl, pH 8,2), then by a regeneration-buffer (20
mM Tris-HCl + 1 M NaCl, pH 8,2) and then by a start-
buffer2 (20 mM Tris-HCl + 150 mM NaCl, pH 8,2). The
volume used for each solution, 5-times than the total
of the column, was pumped at a rate of 5 ml/min.

Each sample prepared was charged into the chroma-
tographic column at a rate of 5 ml/min. until column
saturation and the obtained residue was collected and
frozen. The following fractions separation was ob-
tained by passing through the column 3 volumes of in-
creasing ionic strength NaCl solutions (250 mM, 500
mM, 750 mM, 1 M) buffered at pH 8,1.

Ion-exchange chromatography process was continuously
performed inside a proper cooler in order to maintain
a temperature of about 4°C.

In vitro intestinal fermentations

As it is a matter of processes that occur on the in-

testinal content of large intestine after digestion and ileal adsorption, it is necessary to simulate such effect on the feed to be employed in the fermentation. The feed, comprised of: corn, 38%; barley, 30%; wheat
5 bran, 15%; soya flour, 12%; herring flour, 2%; vitamins and minerals, 3%, is prepared by enzimatic pre-digestion divided in two moments, according to the first part of Vervaeke et al. methodology (1989).

1st – Gastric digestion step: 2 g of feed (ø of the
10 particles < 1 mm) in 40 ml of 0,2% pepsin 0,075 N HCl solution (E.C. 3.4.23.1, P7000; Sigma Chemical, St. Louis, MO, USA) are incubated at 39°C in the stirred bain-marie for 4 hr.

When first passage solution has been adjusted at pH
15 7,5 with 1 N NaOH, it begins the

2nd – intestinal digestion step: wherein an equal quantity of buffer, 40 ml each 2 g of feed is added to 1% pancreatin (P1500 Sigma Chemical, St. Louis, MO, USA) and incubated as in the 1st passage.

20 The buffer was obtained by mixing:

- 500 ml of a solution: 46,5 g Na_2HPO_4 + 49,0 g $NaHCO_3$ + 2,35 g of NaCl + 2,85 g of KCl, first dissolved and then adjusted to 1 liter with ion-exchanged water,

- 5 ml of a 6% $MgCl_2$ solution,

25 - 5 ml of a 4% $CaCl_2$ solution.

Therefore, total 510 ml were then adjusted, still with ion-exchanged water to 2,5 liters and then adjusted at pH 7,5 with 3N HCl (Martillotti et al., 1987).

The pre-digested food was obtained by centrifugation
30 at 3000 x g and 4°C, washed 3 times with ion-exchanged water, intestinal adsorption simulation always eliminated by centrifugation.

The solid residue accumulated on tubes bottom after centrifugation was placed in a stove at 55°C, the re-

quired temperature in order to prevent food active principles denaturation, until drying (about 24 hours), then re-grounded and stored sealed in a freezer.

5 Food analysis before and after pre-digestion

	Whole feed	Pre-digested feed
% humidity	10,77	0,00
% crude protein	17,59	4,19
% crude lipids	4,10	1,98
% crude ashes	3,73	2,95
% NDF	14,08	27,23
% ADF	4,97	11,27
% starch	49,42	50,04

Extraction efficiency 35-40%

15 Caecal inoculum preparation obtained by slaughtering

1st – Withdrawal step: just after the intestine extraction from the carcass, the cecum was bonded at the level of the ileum-caecal valve and cut. From a little hole at cecum head, the content was flown gently in a

20 nylon vessel, from which air was completely discharged, and dipped in water at 39°C into a thermal bag, until the preliminary step carried out in a laboratory.

2nd – Laboratory step: the caecal content was measured

25 by pouring it in a graduate, which was held at 39°C before use like all other vessels to be used in direct contact with the inoculum. When the volume was known, it was mixed with the buffer solution in a 1:2 ratio. The buffer composed of: $NaHCO_3$, 49 g; KCl, 2,85 g;

30 $CaCl_2$ x $6H_2O$, 0,395 g; Na_2HPO_4 x $12H_2O$, 46,5 g; NaCl, 3,5 g; $MgSO_4$ x $7H_2O$, 0,6 g dissolved in 5 l of ion-exchanged water (McDougall, 1948) was adjusted at pH 6,7 with the addition of 3N HCl. This solution was maintained at 39°C in a bain-marie and sparged with

anhydride (CO_2) for 20 min before use. The compound
was then filtered through 8-layer gauzes in a flask,
closed with cotton and gauze and then put again in a
bain-marie under CO_2 for 10 min. In the vessels with
5   the pre-digested feed, heated at 39°C, the freshly
prepared caecal content was poured by way of a cannula
connected to a cylinder, after sparging of CO2 on the
bottom.

Immediately after, the jars were tight-sealed and
10   dipped in a bain-marie at 39°C, where they remained
continuously stirred throughout the fermentation
lenght (48 hours).

Fermentation operational diagram:
- Thesis 1, Control: 20 mg flour + 5 ml inoculum
15   - Thesis 2, BAC +: 20 mg pre-digested feed + 5 ml in-
oculum + pediocin A equal to a final concentration =
160 AU/ml,
- Thesis 3, BAC-: 20 mg pre-digested feed + 5 ml in-
oculum + same fractions of supernatant elution of
20   FBB61-2 Pediococcus pentosaceus corresponding to FBB61
Pediococcus pentosaceus fractions containing pediocin
A. The added quantity produced a proteinaceous concen-
tration equivalent to that produced with pediocin A in
thesis 2.

25   Chemical analysis of feeds and caecal liquid
Diet centesimal analysis was performed according to
AOAC procedure (1990), and the fibrous fractions were
neutral-washed (NDF), acidic-washed (ADF) and the li-
gnine (ADL) according to Van Soest et al., in 1991.
30   Instead, hemicellulose and cellulose were calculated
by the difference between NDF and ADF, and ADF and
ADL. Gross energy was evaluated by bomb calorimeter
(1261 Model; Parr Instrument, Moline, IL, USA).
Cresol concentration determination in samples was per-

formed following the procedure suggested by Birkett et al., 1995.

It emerged from the sperimentation that collected samples during 48 hours fermentation shown a significant
5 reduction in cresol production. Just after a 31 hours fermentative process, the presence of pediocin A in the treatment called BAC+ (in which treatment a producer strain was used) led to a cresol, oncogenic substance, production reduction, (-73.30%; P<0.05) com-
10 pared to treatment with isogenetic mutant (BAC-, (in which treatment it was used a non producer and sensitive strain), while after 48 hours cresol concentration reduction the presence was attested on still more significant values: -87.65%; P, 0.05. The addition of
15 pediocin A to fermentation liquid at a final concentration of 160 AU/ml, led to a very strong putrescine production during the first 4 hours (+203.40% vs BAC-; P,0.001) after which it was stabilized on these values until the end of the test. Spermidine concentration
20 after addition of pediocin A to fermentation liquid always attested on higher values compared to reference control (BAC-), even if from high values recorded on 16th hour (37.58 μmol/L) more reduced values were reached at the end of the test (8.08 μmol/L).
25 In the PROSPECT 1, Figure A, B and C it is reported the effect of pediocin A on polyamines and cresol production, when introduced in fermentation liquid at a final concentration of 160 active units (AU)/ml.
Animals and diet
30 An in vivo test (72 weaned little pigs, seghers breed) monitored the effects of a diet supplemented with the pediocin A producer strain FBB61 Pediococcus pentosaceus (BAC+) in comparison with the BAC- negative control determined from FBB61-2 *Pediococcus pento-*

saceus isogenetic strain. Basic diet was constituted as described in table 1 and 2. From the beginning of the experiment, 56 days lasting, animals (females and gelt males) shown an average live weight (LV) of 7 kg

5   and were 21 days aged. The subjects were divided in 3 groups: 1) CTR control receiving a standard diet, 2) BAC+ receiving coltured FBB61 Pediococcus pentosaceus over a M17 medium (OXOID) (feed/colture broth ratio 2:1), 3) BAC- receiving FBB61-2 Pediococcus pento-

10   saceus grown in the same colture medium and same ratios. From day 0 to day 35 of the test, the animals were fed ad libitum, then rationed at 9% metabolic live weight. On days 0, 14, 35, 56 animals were weighed separetely and growth performances collected,

15   as well as consumption parameters. Variances study for the performance parameters was developped according to GLM (General Linear Model) using SAS ver 6.12 (SAS Institute). Initial live weight was considered as covariance in order to evaluate live weights and daily

20   increases. Used statistical model was the following:
$$Yij = \mu + \tau i + \beta j (xij - x) + \varepsilon ij$$
where: μ = general average; τ = animal effect; β = correction factor for the co-varied; x = examinations average; ε = error.

25   After 35 days test, 6 animals from treatments BAC+ and BAC- were sacrificed for the intestinal morphometric parameters analysis (thickness of small and large intestine's wall, crypts depth, villi thickness, villi length, mucosal tunica, mitotic index, apoptosys in-

30   dex). At the same time, also pancreas was extracted for the same kind of laboratory analysis. The following day, 4 animals of each group were selected in order to test intestinal mucosa functionality, by means of in vivo absorption tests. For this purpose, 2

marker molecules were used: bovine serum albumins
(BSA, 500 mg/kg LW) and sodium-fluorescine (Na-f, 9,4
mg/kg LW). Molecules were dissolved in normal saline
before being introduced directly in the gastric cavity
5   by catheterization. After 0,5, 1, 2, 4, 8 and 24 hr
from administration, 4 ml sampling of blood were taken
for following analysis.

From performed experimentation, it emerged that the
comparison executed between growth performances of two
10   groups receiving two bacterial strains shown interest-
ing aspects (table 3). Just after 35 days from the be-
ginning of the test, even without significant weight
differences, animals receiving FBB61 Pediococcus pen-
tosaceus shown a better use of feeds, as indicated
15   from food capability ratios (FCR), when compared to
their inner controls (BAC-) (1.58 BAC+ vs 1.68 BAC-,
P=0.09). As the food capability did not differ during
the first investigation period (0-14 days), the best
registered trend after 35 days is to be ascribed to a
20   best FCR during the period from day 14th to 35th: BAC+
-5.6% (P=0.08) vs BAC-.

Histological tests shows deeper crypt in animals be-
longing to BAC+ group than those of BAC- group, both
in the proximal and medium intestinal jejunum, sug-
25   gesting in these regions a higher cell proliferation
rate. Conversely, histological test of medial intes-
tine

revealed a lower mitotic index in animal receiving
FBB61 Pediococcus pentosaceus (ATCC 43200) compared to
30   animals receiving FBB61-2 Pediococcus pentosaceus.
This date explains how the greater depth of crypts is
due to a greater secretion activity of cells therein,
rather to their greater proliferation. Relative meas-
urements to intestinal villi structures shown (Table 4

and 5): 1) a potential greater length of villi in intestinal proximal and medium jejunum; 2) a significant increase of brush border thickness, constituted of micro-villi at enterocytes luminal apex; 3) a significantly thicker mucous tunica in comparison with BAC-group animals, both on a proximal and medium jejunum level; a lower cell division rate on a medium jejunum level. The latter remark combined to the potential greater thickness of villi may indicate a reduced intestinal cell turnover, which would not affect the mucosa integrity, however, as demonstrated by in vivo permeability tests. Increasing of brush border thickness of enterocytes, with the potential greater thickness of villi preludes an increased absorbing surface. On a caecal level, a treatment with FBB61 Pediococcus pentosaceus led to a reduced thickness of mucous tunica without affecting other structures, as well as to unchanged pancreatic histological parameters.

Table 1: control diet used into in vivo test. Treated group diets, starting from the same base, included an addition in a 1:2 ratio of colture broth containing FBB61 Pediococcus pentosaceus (BAC+) or FBB61-2 Pediococcus pentosaceus (BAC-).

Ingredients (g/kg feed)

Corn 327.2

Soybean flour 210.0

Soybean oil 20.0

Barley 70.0

Flaked barley 250.0

Fish meal 30.0

Powdered serum 50.0

L-lysine 5.0

DL methionine 1.0

Calcium carbonate 3.0

Calcium phosphate 27.0

Sodium chloride 1.8

Vitaminic mineral mix 5.0

SUPPLIES (per kg of feed)

5   Proteins 179.4

Fats 52.9

Fibre 47.9

Ashes 66.2

Starch 418.5

10   Digestible energy (MJ) 0.811

Net energy (MJ) 0.612

Supplies of vitaminic mineral mix (per kg of feed)

Vitamin A (UI) 15,000

Vitamin D3 (UI) 1,700

15   Vitamin E (mg) 35

Fe 200

Cu 170

Zn 200

Table 2. Diet amino acid profile.

20

	Total AA %	Digestible AA %
Met + Cys	0.73	0.63
Lysine	1.37	1.24
Threonine	0.71	0.60
Tryptophan	0.23	0.19

25   In PROSPECT 2, Table 3 productive performances of ani-
mal are reported. Date are presented as ± mean S.D.
(ADG = average daily increase; FCR= food efficiency).
In PROSPECT 3, Table 4 morphometric analysis effected
over the intestine are reported. Date are presented as
30   ± mean SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.0001$ when
compared to BAC= vs BAC- group animals. MI = mitotic
index.
In PROSPECT 4, Table 5 morphometric analysis effected
over caecum and pancreas are reported. Date are pre-

-17-

sented as ± mean SEM. ** P<0.01, when compared to BAC= vs BAC- group animals. MI = mitotic index.

In PROSPECT 4, Figure D a permeability test is reported. Na-f concentration (μg/ml) in the sampled animals plasma. Date are presented as ± mean SD.

PEDIOCIN-CELL STRUCTURES INTERACTIONS

FBB61 Pediococcus pentosaceus lactic bacterium colture, its pediocin A bacteriocin production and its purification were executed as above discussed. Purificated pediocin A was then labelled using an "ECL protein biotynilation" system (Amersham Pharmacia Biotech, UK) following producer's instructions. Labelled protein detection· was performed using Streptavidin horseradish peroxidase conjugated (HRP) for the detection of the Western blot analysis, or by means of Streptavidin-fluorescein isothiocyanate conjugated (FITC) (Sigma Aldrich, USA) for microscopic examinations.

Pediocin A detection over intestinal mucosa

Swines, rats and mice were sacrificed, small and large intestines were collected and immediately washed in cold PBS solution (pH 7.5). A little portion of jejunum in rat and mouse was reversed, so that the mucosa could be perfectly contacted with PBS solution, while from the swine intestine a 0,25 cm² area was collected. Thus obtained intestine portions were then dipped for 15 min. in a PBS solution containing pediocin A biotynilated (160 AU/ml). Next, in order to remove the protein non-bonded to the mucosa, tissues were washed 2 times in cold PBS. Intestinal portion were then dipped in 2 ml sterile distilled water to remove labelled protein. Tissues were then eliminated. 2 ml solution were then freeze-dried and re-suspended in 40 μl for following analysis by SDS-PAGE.

SDS-PAGE and Western blot

Obtained samples were examined by SDS-PAGE according
to Laemmli (1970) using 3% and 12% acrylamide stacking
gel for running gel. After electrophoresis, the pro-
5   teins isolated from gel were plotted over a nitrocel-
lulose carrier by using a mini Trans-blot (BIO-RAD).
Pediocin A detection was performed by using strepta-
vidin-HRP.

Pediocin-mucus interactions

10   For this test, II type mucines from swine stomach were
used, suspended in PBS solution at a concentration of
1 mg/ml. The same quantity (1 mg/ml) was used and in-
cubated for 15 min. at 37°C in a pediocin A-containing
solution (160 AU/ml). Samples were then centrifugated
15   at 8000xg for 15 min. and the obtained pellet was
washed 3 times with PBS solution. 3 µl of Strepta-
vidin-FITC were added to the pellet, in the meantime
re-suspended in 1 ml of PBS solution. After further 15
min. incubation at room temperature, samples were a-
20   gain centrifugated and pellet washed 3 times with PBS
solution. Swine collected tissues followed the same
iter: a 0,25 cm^2 area was collected and incubated for
15 min. at room temperature in a biotynilated pediocin
A-containing buffer at a concentration of 160 AU/ml.
25   The non-bonded protein was washed away through purges
with PBS solution. Thus obtained samples were analyzed
by using an TMD Nikon inverted microscope provided
with Omega XF100 FITC filter set (Optical-USA). The
used negative control and represented by the same sam-
30   ples not incubated with pediocin. This is to exclude
label interactions with intestinal structures, inde-
pendently from the presence of pediocin A.

Electron microscopy

FBB61-2 Pediococcus pentosaceus cells (non producer

isomutant strain and sensitive to bacteriocin action)
contained in 1 ml of fresh colture were collected by
centrifugation and re-suspended in 300 µl of PBS solu-
tion containing biotynilated pediocin A at a concen-
5   tration of 160 AU/ml. After 2 washes with PBS, la-
belled streptavidin with golden particles (diameter 5
nm) was added. After 10 min. incubation, cells were
washed in PBS. Swines, rats and mice were sacrificed,
small and large intestines were collected and immedi-
10   ately washed in cold PBS solution (pH 7.5). A little
portion of jejunum in rat and mouse was reversed, so
that the mucosa could be perfectly contacted with PBS
solution, while from swine intestine a 0,25 cm^2 area
was collected. Intestine portions thus obtained were
15   then dipped for 15 min. in a PBS solution containing
biotynilated pediocin A (160 AU/ml). Next, in order to
remove the protein non-bonded to the mucosa, tissues
were washed 2 times in cold PBS. At this time, bacte-
rial cells and intestinal tissues were prepared for
20   electron microscope analysis (Jeol JEE 1200 EXII, Jeol
LTD, Tokyo, Japan, at 80 kV). Tissues were also pre-
pared and analyzed by means of Philips XL 30 ESEM
scanning electron microscope.
From the performed experimentation, it has been found
25   that from fluorescence microscope examinations it is
evident that pediocin A interacts with mucus. The ne-
gative control does not show any fluorescence, which
means that streptavidin-FITC does not remain attached
to mucus. Pediocin A recovered from mucus has pointed
30   out the maintenance of an excellent antibacterial ac-
tion, as shown from the presence of inhibition halos
in petri dishes previously seeded with FBB61-2 Pedio-
coccus pentosaceus sensitive strain. The mechanism of
interaction between pediocin A and mucus is not known

yet, but the bacteriocin remains attached also when
two mucopolysaccharide structures are completely satu-
rated by lectins. Protein connection detections to in-
testinal tissues did not display any kind of specific
5   bond with delimitated region by intestine. Conversely,
pediocin A is capable to bond to any structures (large
or small intestine) with no respect of 3 tested animal
species (swine, rat, mouse). An aspecific interaction
but in any case effective is further shown between pe-
10   diocin A and bacterial strain sensitive to its FBB61-2
Pediococcus pentosaceus action. Already after 5
minutes co-incubation, pediocin A molecules are de-
tected close to bacterium cell membrane, both in com-
binations and single molecules. Electron and scanning
15   microscope examinations shown the presence of pediocin
A labelled with streptavidin-gold. More particularly,
also in this case pediocin A seems to remain entrapped
in the mucus covering gastrointestinal structures,
giving protection against GRAM+ pathogens thanks to
20   its preserved antibacterial activity.
Pediocin A has been further characterized as discussed
in following tests #1, #2 and #3.
Test # 1: purification of pediocin A produced by FBB61
P. pentosaceus through tangential flow filtration
25   **(TFF).**
In this test a new method of purification of pediocin
A was tested by tangential flow filtration technique
(TFF). In the usual filtration, a liquid flow to be
filtered has a perpendicular direction based on fil-
30   tering membrane surface, whereas in the TFF the flow
runs driven by a pump, in a tangential direction based
on filtering membrane. Fluid components which are held
and do not pass-through the membrane, as is the case
with TFF, do not accumulate over filter surface but

they are transported by the flow, preventing in this
way the pore obstruction of the membrane.

In the filter modulus, a separation between filtrate
and retentate occurs; the filtrate is collected, while
5   the retentate is re-introduced in the supply vessel to
cyclically proceed the filtration and to increase
yields in terms of solute purification and concentra-
tion to be recovered.

Colture broth to be subjected to tangential flow fil-
10   tration (TFF) was obtained by incubating FBB61 P. pen-
tosaceus in a M17 broth + 1% (w/v) glucose at 39°C for
20 hours at 38°C. At the end of the incubation, the
colture broth was centrifugated at 10,000 rpm for 5
minutes, the supernatant collected and filtered
15   (Stericup, Millipore Corporation, Bedford, Massachus-
sets) through Durapore® membranes with 0,45 mm pore
diameter. For TFF, a flat filtering modulus was used
(Pellicon® XL filter, Millipore Corporation, Bedford,
MS, USA), with a modified polyether sulfone Biomax®
20   membrane and NMWL (Nominal Molecular Weight Limit) of
100 kDa (Fig. 15).

TFF filtering modulus was prepared according to pro-
ducer's instructions. Filtered colture broth was
placed in the filtering modulus by means of a peri-
25   staltic pump (Minipuls 3, Gilson Italia Srl, Milan,
Italy) at a 20 ml/min flow. Filtered colture broth,
filtrate and preparation solutions were held in an ice
bath throughout the filtration lenght. After reducing
the supply solution volume (2,000 ml) at about a quar-
30   ter of initial volume (530 ml), TFF was interrupted
and the two solutions thus obtained, the supply solu-
tion, that is retentate (R2), and the filtrate (F2)
were frozen at   -20°C.

TEST # 1 RESULTS

By using a filter with NMWL (Nominal Molecular Weight Limit) of 100 kDa for the tangential flow filtration process, it was believed to recover pediocin A in the filtrate (F), i.e. the crossing filter component. Con-
5 trary to all expectations, the inhibiting activity to-wards the indicator strain (FBB61-2 P. pentosaceus) was found in the retentate (R) containing the sub-stances not able of passing-through the used filtering membrane. By considering that the molecular weight of
10 pediocin A is 80 kDa, probably this result has to be ascribed to the formation of aggregates of pediocin A, given its hydrophobic nature (Piva and Headon, 1994) which is not able to pass through the meshes of the filter used.
15 The purification of 2,000 ml colture broth with the tangential flow filtration method enables to reach a high efficiency purification (3,55 times the initial colture). The specific activity, hence the ratio be-tween arbitrary units and proteinaceous content.
20 (AU/mg) of the sample presents a value of 21.88 AU/mg vs initial 6.16 AU/mg. Further, the total recover of the activity initially present in the filtered super-natant of the existent colture broths inside the ini-tial colture broth (100%) was of particular impor-
25 tance, whereas previous purification methodologies also led to an average diminution of about 20-30% in the first purification steps.
The purification by tangential flow filtration re-quires considerably lower periods compared to purifi-
30 cation with conventional method, by thus facilitating manual procedures and lowering possible error sources and so losses of pediocin A content, and it proves to be a valid purification method in expectation of high quantity production of bacteriocins, particularly of

pediocin A.

Test # 2: determination of pediocin A thermal sensitivity

Pediocin A was obtained by incubating FBB61 P. pentosaceus in a M17 broth + 1% (w/v) glucose at 39°C for 20 hours at 38°C. At the end of the incubation, the colture broth was centrifugated at 10,000 rpm for 5 minutes, the supernatant collected and filtered (Stericup, Millipore Corporation, Bedford, Massachussets) through Durapore® membranes with 0,45 mm pore diameter. Further, the filtered supernatant was concentrated through dialysis against polyethylene glycol of 20000 Da molecular weight (PEG 20000, Sigma Chemical, St. Louis, MO, USA). For this operation, dialysis tubes with 12000/14000 MW cut-off (Spectra/Pro 4, Spectrum, Houston, Texas, USA) were employed. The process was carried out at a temperature of 4°C for a whole night. To adjust pH values close to neutral, the dialysed thus obtained was subjected to a second dialysis against a PBS solution (137 mM NaCl + 2,7 mM KCl + 4,3 mM $Na_2HPO_4.7H_2O$ + 1,4 mM KH_2PO_4) for 24 hours at 4°C, and designated D1+PBS.

Thermal sensitivity of pediocin A thus produced and semi-purified was evaluated by exposing aliquots of D1+PBS preparation at increasing temperatures (45°C, 55°C, 65°C, 75°C, 85°C) for increasing time intervals (5 min., 15 min., 30 min., 45 min., 60 min.). Four aliquots of 1 ml pediocin A D1+PBS preparation were distributed in eppendorf tubes and dipped in water for each temperature-time combinations. After exposition to the temperature for the established time intervals, each sample was freezed at − 20°C before evaluating pediocin A activity by wells technique.

TEST #2 RESULTS

The performed test allowed to confirm thermal sensitivity of pediocin A, which does not show any inhibition of FBB61-2 P. pentosaceus indicator strain in the activity evaluation by means of wells technique, if it
5 has been previously subjected to a treatment at 65°C for 45 minutes (Tab. 6). Treatments at 65°C for lower time intervals than 45 minutes and treatments at lower temperatures than 65°C for all considered time intervals did not decrease pediocin A activity. Higher tem-
10 peratures treatments than 65°C, for each considered time interval decreased the inhibiting activity of pediocin A below detection limit of the selected technique.

Thanks to its characterization, pediocin A may be used
15 in all those preparations whose production technology involves the attainment of high working temperatures, not higher than 65° for not longer than 30 minutes.

Tab. 6. AU/ml detected with wells technique over pediocin A samples exposed to thermal treatment.

20

	45°C	55°C	65°C	75°C	85°C
5 min.	1,600	1,600	1,600	0	0
15 min.	1,600	1,600	1,600	0	0
30 min.	1,600	1,600	1,600	0	0
45 min.	1,600	1,600	0	0	0
60 min.	1,600	1,600	0	0	0

Test #3: contact tests between pediocin A and Clostridium perfringens

Pediocin A antimicrobial activity over the C. perfringes growth was evaluated by exposing Clostridium
25 botulinum bacterium to increasing quantities (20, 40, 80, 160, 320 AU/500µl) of bacteriocin itself for a predetermined time interval of 60 minutes. For this test, pediocin A preparation obtained by tangential

filtration was used, as in the above discussed "Test #2".

TEST #3 RESULTS

It has been detected a significant reduction ($P<0.05$)
5 of C. perfringens cells number recovered at the end of 60 minutes of contact with the solution containing a greater quantity of pediocin A (320 AU/500 µl) based on cells number recovered after 60 minutes residence time in a normal saline. Treated sample with 320
10 AU/500µl shows a reduction of colonies-forming units (CFU) recovered at 67% based on a non-treated sample. Such reduction is not present in other examined samples.

Tab. 7. Contact test results between Clostridium per-
15 fringens and pediocin A. AU = arbitray units of pedio-
cin A existent in 500µl; * = no.=3; to different let-
ters correspond significantly different results ($P<0.05$).

AU	UFC/500µL*
0	8.00±2.83[a]
320	2.67±0.58[b]
160	9.50±0.71[a]
80	8.00±3.46[a]
40	10.00±1.73[a]
20	12.00±2.83[a]

20 Further, from the contact tests by wells technique it has been possible to evaluate the Clostridium perfrin-
gens bacterium sensitivity against pediocin A antim-
icrobial action, by comparing it to that of FBB61-2 Pediococcus pentosaceus standard indicator strain.
25 This text allowed to establish that C. perfringens sensitivity is 160-fold higher than FBB61-2 P. pento-
saceus.